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(54) Title: ROOT KNOT NEMATODE RESISTANCE

(57) Abstract

In order to provide plants which exhibit resistance to infection by root knot nematodes identification is made of a gene which is expressed at the nematode feeding site of an infected plant. The promoter of the feeding site gene is isolated and associated with a sequence which encodes a molecule inimical to root knot nematode infection. A further plant is transformed with the so constructed chimaeric gene.

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Root Knot Nematode Resistance

The subject invention relates to resistance in plants to deleterious effects of infection by root knot nematode.

Root knot nematodes (*Meloidogyne* spp) are major pathogens of many crop plants, for example vegetables, food legumes, tobacco, tomato, water melon, grape, peanut and cotton.

Chemical control, cultural practices and the use of resistant varieties are the chief approaches to nematode control which are currently available and they are often used in an integrated manner against root knot nematodes. There is a requirement for improvement in the nematode control because these current approaches offer inadequate crop protection. Nematicides are of questionable environmental status and they are not always efficacious. Cultural control imposes hidden losses on growers in several ways. The wide host range of root knot nematodes limits the availability of economically satisfactory non-host crops. Effective resistant cultivars are frequently unavailable and those that the grower can use are sometimes out-performed by susceptible cultivars at low root knot nematode densities. Also resistance may be lost in the high soil temperatures that occur in tropical and sub-tropical environments.

When a root knot nematode invades the root of a plant it migrates intercellularly until it reaches the

root meristem. Pharyngeal gland secretions are then injected through the stylet of the nematode into cells in the region of the meristem. This causes the normal development of these cells to be disrupted, whereby nuclear division occurs without the occurrence of cell division. There are thus formed multi-nucleate cells, known as "giant cells". Accompanying the formation of the giant cells is the expansion of surrounding cells, known as hypertrophic cells, which the nematode does not attack directly by stylet penetration. The giant cells and the surrounding hypertrophic cells together constitute the feeding site of root knot nematodes. The observed knot formed on the infected root consists of such giant cells and the accompanying hypertrophic cells which are the result of a multiplicity of nematode infections. The mechanism of the production of giant cells is similar in all susceptible plant species.

Following giant cell induction, the root knot nematode loses locomotory ability as feeding by the nematode on the giant cell proceeds, and the nematode becomes committed to feeding, development and reproduction at the feeding site.

In International Patent Publication WO 92/04453 there is described a method of control of root cyst nematodes. In a paper entitled "Gene Expression in Nematode-Infected Plant Roots" by Gurr et *al* (1991), there is described, in relation to potato cyst nematodes a method for obtaining cDNA from mRNA which is found in

the syncytium of a plant which has been infected by the root cyst nematode.

The subject invention provides a method of producing root knot nematode resistant plants, wherein in respect of a root knot nematode infected plant there is identified a gene which is expressed in the giant cells and/or the accompanying hypertrophic cells of root knots of the plant, the promoter of said gene is taken and fused with a coding sequence to provide a chimaeric gene which encodes a molecule which is inimical to one or more of 1. root knot giant cells, 2. root knot hypertrophic cells and 3. root knot nematodes, and a further plant is transformed with said chimaeric gene.

Plants to which may be imparted root knot nematode resistance in accordance with the subject invention include vegetable plants, food legumes, tobacco, edible fruit plants, edible nut plants and cotton. Thus, for example, in respect of vegetable plants the subject invention may be applied to carrot plants and in respect of fruit plants it may be applied to tomato plants.

In that the mechanism of production of giant cells is similar in all susceptible plant species, the inventive method is, in fact, applicable to all such species which are also transformable in accordance with the transformation step of the method.

The inventive method is applicable in respect of *Meloidogyne* species including but not limited to *M.incognita, M.javanica, M.arenaria* and *M.hapla*.

The gene identified and selected from an infected plant is preferably one the expression of which takes place not before the nematode has substantially lost locomotory ability.

Sequences (in the chimaeric gene) to be expressed under the control of the said promoter include one or more of:-

1. A coding sequence for a molecule that causes necrosis of giant cells and/or hypertrophic cells.
2. A coding sequence for a molecule that causes necrosis of a root knot nematode.
3. A coding sequence for any of a number of enzymes which are active to impair plant cell metabolism.
4. The antisense of the feeding site specific gene.
5. Antisense of the coding sequence for enzymes critical to plant cell metabolism.

It will generally be necessary to ensure that a gene is selected which is a gene which is expressed in only the giant cells and/or the accompanying hypertrophic cells, since if a gene was expressed at another site, the expression product of the chimaeric gene produced at the other site might adversely affect the transformed plant.

It is an advantage of the present invention that root knot nematode resistance can be imparted to plants without the need to produce constitutively an anti nematode infection product as listed above at 1-5.

A preferred procedure for carrying out the subject invention will now be described.

Growth and Infection of tobacco plants

Seeds of C319 tobacco are germinated on Fisons F1 compost under conditions as follows. Light intensity of 4500 to 5000 lux, with 16 hour periods of light alternating with 8 hour periods of darkness, and temperatures between 20°C and 25°C. After *c.* 3 weeks seedlings are gently washed in tap water to remove soil and transferred to pouches (2 plants per pouch; Northrup-King) and grown for a further week in a Conviron at 25°C and with a light intensity of 5500 lux for 16 hour periods alternating with 8 hour periods of darkness. Roots are lifted from the back of the pouch and supported with Whatman GF/A glass fibre paper at their tips. Three day old nematodes (*M. javanica*) are then delivered to the tips of these roots in 10µl (50 nematodes) aliquots and a second piece of GF/A paper is placed on top to fully encapsulate the root tip. Following 24 hours post infection, the GF/A paper is removed to ensure synchronous infection. Following 3 days post infection the knots are dissected out (leaving healthy root and root tip tissue behind) and frozen immediately in liquid nitrogen. Approximately 0.5 to 1g of infected root tissue can be harvested from 80 inoculated plants.

Staining for visualisation of nematodes in infected roots

To establish the quality of the infection the number of nematodes (infecting) per root tip is determined. Roots are harvested from 3 day post infected plants and

immersed for 90 seconds in lactophenol containing 0.1% Cotton Blue at 95°C. Following a 5 second rinse in water, the roots are placed in lactophenol at room temperature (RT) for 3 - 4 days to clear. Stained nematodes are then visualised using light microscopy.

RNA isolation from healthy and infected root tissue

Root tissue is ground to a fine powder in a chilled (liquid nitrogen) pestle and mortar. About 100mg aliquots are then transferred to similarly chilled Eppendorf tubes and 300µl of hot phenol extraction buffer added (50% phenol, 50% extraction buffer : 0.1M lithium chloride, 0.1M Tris-HCl pH8.0 (RT), 10mM EDTA, 1% SDS) and incubated at 80°C for 5 mins. An equal volume of chloroform is then added and the homogenate microfuged for 15 minutes at 4°C. The aqueous phase is then extracted with 600µl of phenol/chloroform and microfuged as above. Following this, the aqueous phase is again removed and then the RNA precipitated with an equal volume of lithium chloride at 4°C overnight. The precipitate is pelleted by microfuging for 15 minutes at RT and washed in 70% ethanol. The pellet is then lyophilised, resuspended in DEPC treated water and assayed using a spectrophotometer. RNA quality is assessed by denaturing gel electrophoresis. (Adapted from Shirzadegan *et al* 1991).

Subtractive cloning of infection specific cDNAs

Poly(A)$^+$ RNA (mRNA) is isolated from 200µg total RNA samples from healthy and infected C319 root tissue using magnetic oligo dT Dynabeads according to the

manufacturer's instructions. First strand cDNA synthesis is performed *in situ* on the Dynabead bound poly $(A)^+$ fraction from the healthy tissue. This is the Driver DNA. First and second strand synthesis is performed *in situ* on the Dynabead bound poly $(A)^+$ fraction from the infected tissue. This is the Target DNA. All cDNA reactions are carried out using Pharmacia's cDNA synthesis kit and according to the manufacturer's instructions. Three oligonucleotides, SUB21 (5'CTCTTGCTTGAATTCGGACTA3'),SUB25(5'TAGTCCGAATTCAAGCAAGAG CACA3') (sequences from Duguid & Dinauer, 1990) and LDT15 (5'GACAGAAGCGGATCCd$(T)_{15}$3') (O'Reilly *et al*, 1991) are kinased with T4 polynucleotide kinase according to Maniatis *et al*, (1982). SUB21 and SUB25 are then annealed to form a linker which is then ligated to the target DNA with T4 DNA ligase according to King & Blakesley (1986). Following this, the beads carrying the Target are washed extensively with TE and the second strand of the cDNA eluted at 95°C in 5xSSC.

The RNA bound to the Dynabead bound Driver DNA is removed by heat and the eluted Target DNA hybridised to the Driver DNA at 55°C in 5 x SSC for 5 hours. Non-hybridising Target DNA is separated from the bead bound driver DNA at room temperature following the manufacturer's instructions, following which, hybridising Target DNA is similarly separated from the bead bound Driver DNA at 95°C. The RT eluted Target DNA is then added back to the Driver DNA and the hybridisation repeated. This process is repeated until the amount of

Target hybridising to the Driver no longer exceeds the amount that does not hybridise. DNA concentrations are established using Invitrogen's DNA Dipstick in accordance with the manufacturer's instructions.

Aliquots of the final RT eluted fraction are used in PCR amplification (Eckert *et al*, 1990) to generate double stranded cDNA for cloning into a plasmid vector. Amplification of the Target DNA is achieved using the primers SUB21 and LDT15 and a Hybaid Thermal Cycler according to the conditions described by Frohman *et al*, 1988. The PCR products are then ligated into *Sma1* digested pBluescript vector according to King & Blakesley (1986).

Screening of the subtractive library by Reverse Northern analysis

Recombinants are identified by colony PCR (Gussow & Clackson, 1989). The amplified inserts are Southern blotted in triplicate onto Pall Biodyne membranes as described by the membrane manufacturer. Prehybridisation and hybridisation are carried out with the same temperature and buffer which are 42°C and 5 x SSPE,0.05% BLOTTO,50% formamide. These are hybridised separately to cDNA probes (see below) from healthy and infected tissue and to a probe comprising amplified Target DNA from the final subtraction. Clones that show a hybridisation signal to the infected cDNA probe only or that show a hybridisation signal to the subtracted probe but not the cDNA probes are selected for further analysis.

cDNA probe generation

To achieve high specific activity probes for differential screening, cDNA synthesis is conducted 'cold' on total RNA and the synthesis products then labelled by oligolabelling. Samples of 10μg total RNA from healthy and infected tissue are first treated with 2.5 units DNase 1 at 37°C for 15 minutes. The DNase is then denatured at 95°C for 10 minutes before cDNA synthesis is performed (standard Pharmacia protocol). The RNA is then removed in the presence of 0.4M sodium hydroxide for 10 minutes at RT and the DNA purified through a spun Sephacryl 400HR column. cDNA yield and concentration are determined using DNA Dipsticks (Invitrogen). The cDNA products are then labelled as for Pharmacia's standard oligolabelling protocol (*c.* 35ng/probe).

Northern blotting

To determine the expression profile of the cDNAs selected from the Reverse Northerns in the different tissues of the plant, the clones are used as probes in Northern analysis of either total or poly (A)+ RNA from healthy and infected roots, stems, leaves and flowers. Total RNA blots comprise 25μg RNA per lane whilst poly (A)+ blots comprise 0.5 to 1μg RNA per lane. The RNA is electrophoresed on formaldehyde gels and blotted onto Pall Biodyne B membrane as described by Fourney *et al* (1988). Probes are labelled and hybridised to blots as described above.

Southern blotting

To determine whether the cDNAs are of plant or nematode origin, C319 and *M.javanica* DNA are prepared as described by Gawel & Jarret, (1991). Southern blots are prepared comprising 10μg *Eco*RI and *Hind*III digested DNA per lane. The blots are hybridised to oligolabelled probes as described above.

In Situ hybridisations

To determine the locality of expression of the cDNAs of interest at the feeding site, *in situ* hybridisations are performed. Tissue from infected and healthy roots are embedded in wax, sectioned and hybridised to the probes as described by Jackson (1991).

Isolation of 5' termini of mRNAs

The 5' termini of the RNAs of interest are determined prior to the isolation of their promoter sequences. This is achieved by using 5' RACE as described by Frohman *et al*, (1988).

Isolation of promoter regions

The promoter regions of the genes of interest are isolated by a process termed Vector-Ligated PCR. 100ng samples of restriction endonuclease digested C319 genomic DNA are ligated for 4 hours at RT (King & Blakesley, 1986) with 100ng samples of pBluescript (digested with a restriction enzyme producing compatible termini). Typically, enzymes used are *Eco*RI, *Bam*HI, *Hind*III, *BGl*II, *Xho*I, *Cla*I, *Sal*I, *Kpn*I, *Pst*I, and *Sst*I. PCR is then performed on the ligations using a vector primer such as the -40 Sequencing primer and a primer complementary to

the 5' terminus of the mRNA. The PCR products are then cloned and sequenced. If necessary, the process is repeated with a new primer complementary to the 5' terminus of the promoter fragment to ensure that the control sequences of the promoters are isolated.

Construction of chimaeric genes in binary plant transformation vectors

The isolated promoters are ligated 5' to a sequence which is a sequence of one of the classes 1. - 5. as detailed hereinabove, examples being the antisense of the gene itself (class 4.) or the barnase gene (Hartley *et al*, 1972) (class 1. and/or class 3.). These are constructed in binary vectors (Bevan, 1984).

Transgenic plant production

Transgenic plants, for example tobacco, may be produced by the standard *Agrobacterium* mediated leaf disc method described by Horsch *et al* (1985), thus to provide root knot nematode resistant plants. Seeds or other propagules of plants the product of the subject invention can be stored for future use.

As will be realised by those skilled in the art, with some classes of plant it may be appropriate or ncessary to transform the plant by use of a method other than an *Agrobacterium* mediated method.

As will also be realised by those skilled in the art, upon root knot nematode infection of plants the product of the subject invention the plants no longer suffer the said deleterious effects of such infection and the reproductive capacity of the root knot nematodes is

impaired so that the population of root knot nematodes in the soil at the location of the plants is reduced to an economically insignificant size.

Root knot nematode resistance may be imparted in accordance with the subject invention to all root knot nematode susceptible monocotyledonous, dicotyledonous, herbaceous and woody plant species.

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CLAIMS

1. A method of producing root knot nematode resistant plants, wherein in repect of a root knot nematode infected plant there is identified a gene which is expressed in the giant cells and/or the accompanying hypertrophic cells of root knots of the plant, the promoter of said gene is taken and fused with a coding sequence to provide a chimaeric gene which encodes a molecule which is inimical to one or more of 1. root knot giant cells, 2. root knot hypertrophic cells and 3. root knot nematodes, and a further plant is transformed with said chimaeric gene.
2. A method according to Claim 1, wherein said molecule is a molecule effective to cause necrosis of giant cells and/or hypertrophic cells.
3. A method according to Claim 1, wherein said molecule is a molecule effective to cause necrosis to a root knot nematode.
4. A method according to Claim 1, wherein said molecule is that of an enzyme active to impair plant cell metabolism.
5. A method according to Claim 1, wherein said molecule is the antisense RNA of said gene identified from said infected plant.
6. A method according to Claim 1, wherein said molecule is the antisense RNA of a gene encoding an enzyme critical to plant cell metabolism.

7. A root knot nematode resistant plant the result of carrying out a method according to any one of the preceding claims.

8. A plant according to Claim 7 which is a plant of the group consisting of vegetable plants, food legumes, fruit plants, nut plants and fibre crop plants.

9. A plant according to Claim 7 and being a tobacco plant.

10. A plant according to Claim 8 and being tomato plant.

11. A plant according to Claim 8 and being carrot plant.

12. A plant according to Claim 8 and being cotton.

13. A propagule of a plant according to Claim 7.

14. A chimaeric gene comprising a coding sequence which encodes a molecule which is inimical to one or more of 1. root knot giant cells, 2. root knot hypertrophic cells and 3. root knot nematodes, said gene further comprising a promoter sequence which is operable to cause expression of said coding sequence in root knot giant cells and/or root knot hypertrophic cells.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00514

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; A01H1/02; A01H5/00; A01N63/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H ; A01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
P,X	WO,A,9 221 757 (PLANT GENETIC SYSTEMS) 10 December 1992 see the whole document ---	1-14
P,X	WO,A,9 204 453 (UNIVERSITY OF LEEDS) 19 March 1992 see the whole document ---	1-14
A	THE PLANT JOURNAL vol. 1, no. 2, September 1991, pages 245 - 254 SIJMONS, P.C., ET AL. 'Arabidopsis thaliana as a new model host for plant parasitic nematodes' see page 252, right column, paragraph 2 ---	1-14
A	EP,A,0 298 918 (CIBA-GEIGY) 11 January 1989 see column 6 - column 7 ---	1-14

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 JUNE 1993	13. 07. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 113, 1990, Columbus, Ohio, US; abstract no. 127723, JUN, W. 'Preparation of transgenic plants for the control of virosis' see abstract & CN,A,1 033 645 (FAMING ZHUANLI SHENQUING GONGKAI SHUOMINGSHU) 5 July 1989 ---	1-14
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E	WO,A,9 310 251 (MOGEN) 27 May 1993 see the whole document -----	1-14

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO-A-9204453	19-03-92	AU-A- 8502391	30-03-92
EP-A-0298918	11-01-89	AU-B- 620039 JP-A- 1037294	13-02-92 07-02-89
WO-A-9306710	15-04-93	None	
WO-A-9310251	27-05-93	None	